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Adipocytes Enhance the Proliferation of Human Leiomyoma Cells Via TNF-α Proinflammatory Cytokine

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Abstract

Objective: Obesity is a well-documented risk factor for uterine leiomyoma with a major impact on women health and health care system of the nation. Obesity is associated with increased secretion of adipokines that significantly influence growth and proliferation of tumor stroma and malignant cells. Adipokines, such as tumor necrosis factor α (TNF-α), are produced in the adipose tissue with concomitant expression in other organs and tissues. Increased and sustained cytokine production is associated with alterations in cell growth and differentiation. We, therefore, explored the influence of human adipocytes (SW872 cells)–mediated biological humoral factors on human uterine leiomyoma (HuLM) cells. **Methods:** We measured cell proliferation and expression of cell-proliferating proteins (proliferating cell nuclear antigen [PCNA], cyclin D1, and B-cell lymphoma 2 [BCL-2]) in human leiomyoma cells cocultured with SW872 cells. SW872-conditioned media was neutralized for TNF-α and proliferation of HuLM cells was observed along with antiapoptotic marker, BCL-2, using Western immunoblot. **Results:** We found that both SW872-conditioned media and coculture with SW872 cells increased HuLM cell proliferation significantly ($P < .05$). We determined that this effect was associated with the upregulation of specific markers for proliferation, such as PCNA, cyclin D1, and BCL-2 ($P < .05$). Furthermore, the addition of neutralizing antibodies, anti-TNF-α, to SW872-conditioned media reversed the proliferation of leiomyoma cells and induced apoptosis as indicated by the reduced expression of antiapoptotic marker BCL-2. **Conclusions:** SW872 cells secrete TNF-α, which is associated with a proliferative gene profile in HuLM cells and may play a role in initiation and/or progression of uterine leiomyoma.

Keywords

obesity, uterine leiomyoma, coculture, adipokines

Uterine leiomyomas are benign tumors characterized by increased cell growth and deposition of extracellular matrix. They are the most common indication for hysterectomy in the United States.[1] We and others have shown that the risk of uterine leiomyomas is about 3 to 4 times higher in African American women than in Caucasian women.[2–4] Obesity is another malady afflicting the nation at an epidemic level. According to the National Health and Nutrition Examination Survey III, two thirds of the adults in the United States are overweight, of whom one thirds are obese. Furthermore, obesity is one of the important risk factors for uterine leiomyoma.[5–7] Increase in body mass index increases the risk of developing leiomyoma 2 to 3 times in women.[5]

Obesity is characterized by a chronic state of inflammation and secretion of specific biological factors called adipokines. Increase in adiposity leads to dysregulated adipokine levels and an activation of inflammatory signaling pathways resulting in pathogenic outcomes including cancers.[8–12] The adipose tissue of obese mice and humans produces proinflammatory cytokines, chemokines, and peptides, including tumor necrosis factor α (TNF-α),[13] transforming growth factor β (TGF-β),[14] interleukin 6,[15] and monocyte chemoattractant protein 1.[16] Increased expression of TNF-α has been reported in adipocytes, experimental obese models of animals, and in humans.[11,13,17] Serum levels of TNF-α are higher in obese rodents and women.[11,18] Tumor necrosis factor α (TNF-α) is involved in the regulation of adipokines level[19] and plays an important role in hemorrhagic necrosis of tumors as well as in cell growth regulation, differentiation, inflammation, tumor metastasis, and autoimmune diseases.[20] Obesity-related inflammation is known to induce neoplasm, but the ability of the inflammatory cells to induce early stages of tumorigenesis and later metastasis is not known.

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Despite numerous reports on obesity as a major risk factor for uterine leiomyoma, its exact role in the initiation and growth of leiomyoma is not clear. Existing literature suggests that growth of uterine leiomyoma is hormone dependent with enhanced sensitivity to estrogen as evidenced by growth during the premenopausal age and its shrinkage after menopause.[21] Evidence indicates that hormones exert their growth stimulatory effect through intermediaries like cytokines and growth factors.[22] Abnormal production of these intermediaries may be responsible for increased cellular growth and hypertrophy.

To better understand the biological association of obesity and uterine leiomyoma, we studied the interaction of adipokine-secreting human adipocytes (SW872) with human uterine leiomyoma (HuLM) cells. It is probable that the dysregulation of adipokine levels during obesity may contribute to the development of uterine leiomyomas. With this rationale, we used the invitro cell culture model to investigate cell growth in HuLM cells using SW872-conditioned media, and we also cocultured the SW872 and HuLM cells. To our knowledge, this is the first study evaluating the effect of adipocyte cells on leiomyoma cell proliferation using coculture.

Materials and Methods

Reagents

Neutralizing antihuman TNF-α antibody was purchased from R&D systems (Minneapolis, MN). Primary antibodies for B-cell lymphoma 2 (BCL-2), cyclin D1, and proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). All other cell culture reagents, including CyQuant Cell Proliferation Assay Kit, Dulbecco modified Eagle medium (DMEM), DMEM F12, fetal bovine serum (FBS), penicillin, and streptomycin, were purchased from Invitrogen (Carlsbad, California).

Cells

Human liposarcoma SW872 cell line has been used in earlier studies as a human adipocyte cell model due to its unique physiological response similar to mature adipocytes.[23–25] Compared to mouse 3T3-L1 adipocytes, these cells have the advantage of being human in origin and do not require an incubation cocktail for growth. This cell line synthesizes and secretes apolipoproteins, as well as constitutively expresses several important adipocyte genes, such as PPAR-α, PPAR-γ, lipoprotein lipase, and adipsin.[26] The cell line was obtained from American Type Culture Collection (Manassas, Virginia). Cells were maintained in DMEM/F12 with 10% FBS and 1% penicillin and streptomycin.

Human immortalized leiomyoma (HuLM) cells were a kind gift from Dr Darlene Dixon (National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina).[27] The cell line was cultured and maintained in smooth muscle cell basal medium (SmBM) containing 5% FBS, 0.1% insulin, 0.2% hFGF-β (human Fibroblast Growth Factor-beta), 0.1% GA-1000 (Gentamycin-Amphotericin 1000), and 0.1% hEGF (human Epidermal Growth Factor) (Lonza, Walkersville, Maryland).

Cell Culture

In the transwell system, HuLM cells were cocultured using transwell inserts with SW872 cells on 0.4-μm porous membrane (Corning, Lowell, Massachusetts). After incubation for 2 to 6 days, HuLM cells were harvested from the 24-well culture plate for proliferation assay or Western blot analysis. Briefly, the coculture was set up by plating 1×10^4 SW872 cells in transwell insert and, after 4 hours of equilibration, the media was replaced with FBS-free media. The inserts were further incubated for 12 hours at 5% CO_2. Meanwhile, the HuLM cells were plated at 4×10^3/well per mL into sterile 24-well plates. The inserts with SW872 cells were placed on the subconfluent HuLM cells with sufficient volumes of FBS-free SW872 medium in the insert and the wells of the 24-well plate. After 6 days of coculture, the HuLM cell lysates were harvested using cell lysis buffer for protein quantification and Western blot analysis as well as cell proliferation assay using CyQuant kit (Invitrogen).

Conditioned Media

SW872 cells were grown to 80% confluence. The media was collected, centrifuged, and filtered to remove cell debris. It was diluted 2-fold, 4-fold, and 10-fold using unconditioned media before adding to the HuLM cells. For the antibody neutralization experiments, SW872 cells were grown to 80% confluence. The cells were then starved by replacing their medium with FBS-free media and incubation continued for 48 hours. The FBS-free media was then collected, filtered, diluted to 1% and treated with 1 ng/mL of anti-TNF-α antibody for 1 hour at 37°C. Later, this antibody-neutralized media was added to HuLM cells with appropriate controls.

Colorimetric Assay

CyQuant assay was done to determine cell proliferation in coculture as well as conditioned media–cultured HuLM cells per manufacturer's instructions. Briefly, at days 2, 4, and 6 of coculture or conditioned media treatment, the culture plates were gently inverted to aspirate the medium from the wells and then washed carefully with phosphate-buffered saline (PBS). The plates were immediately frozen at −70°C for 1 hour. The plates were then thawed at room temperature and 200 μL of CyQuant GR dye per cell lysis buffer was added to each well and mixed gently. The plates were incubated for 5 minutes at room temperature protected from light. The sample fluorescence was measured using a fluorescence microplate reader with filters set at 480 nm excitation and 520 nm emission.

Western Blot

After coculture, the HuLM cells were harvested and lysed with a lysis buffer (Cellytic-M; Sigma, St Louis, Missouri)

containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, Indiana). Protein concentration was determined by bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific, Inc, Rockford, Illinois). The samples were diluted with 4× sodium dodecyl sulfur (SDS) loading buffer containing β-mercaptoethanol. Equal amounts of protein (10 μg) were separated on SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, Massachusetts). Proteins were detected by immunoblotting followed by ECL chemiluminescence detection (Amersham Biosciences, Piscataway, New Jersey). Chemiluminescence signals were detected by a luminoimage analyzer SRX-101A (Konica Minolta, Ramsey, New Jersey). Membranes were immunoblotted with the primary antibody against PCNA (1:500), BCL-2 (1:500), and cyclin D1 (1:500). After washing, membranes were incubated with horseradish peroxidase (HRP)–conjugated secondary antibody (1:5000; Santa Cruz Biotechnology). Western blot with anti-β-actin antibody (1:5000) was used for loading control. The intensity of each protein band was determined using a scanning densitometer (Alpha Innotech Imager, Santa Clara, California) and later normalized against the values obtained from β-Actin.

Statistical Analysis

All data are presented as means ± standard error (SE) of all values obtained from 3 to 4 replicate wells repeated at least 3 times. Differences between groups were analyzed using Student *t* test. $P \leq .05$ was considered statistically significant.

Results

Adipocyte-Conditioned Media Enhances Proliferation of Human Leiomyoma Cells

To evaluate the effect of SW872-conditioned media on HuLM cells, SW872 cells were grown to 80% confluence in T200 flasks for the preparation of conditioned media. Varying dilutions of conditioned media was added to HuLM cells grown to 30% confluence in a 96-well tissue culture plate. On day 6, the cell proliferation in HuLM cells was measured using CyQuant cell proliferation kit.

As shown in Figure 1, the control group (cells growing without conditioned media) shows the least number of cells. On the other hand, adipocyte-conditioned media at 25% and 10% concentration showed a 16% ± 0.04% and 20% ± 0.06% induction in human leiomyoma cell proliferation, respectively ($P < .05$). To confirm this result, we repeated the experiment using a coculture method without direct cell-to-cell contact.

Coculture With Human Adipocytes Enhances Proliferation of Human Leiomyoma Cells

We further evaluated the potential humoral interaction between SW872 and HuLM cells using a transwell coculture system.

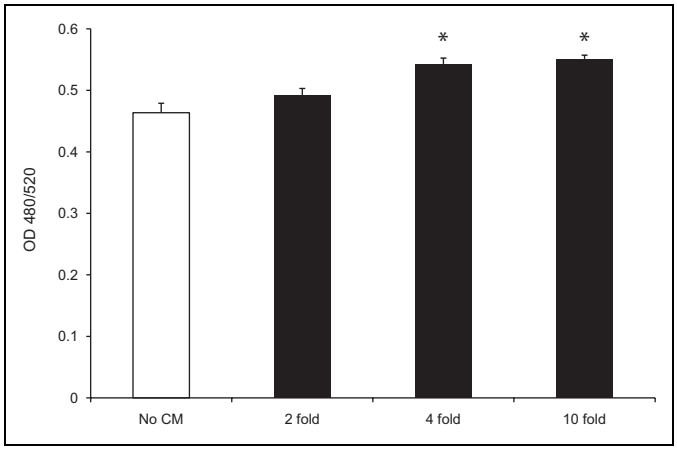

Figure 1. Effect of SW872-conditioned media on proliferation of HuLM cells. HuLM cells were cultured in a 96-well cell culture plate and treated with conditioned media which was diluted from 2- to 10-fold concentrations. Cell proliferation in HuLM treated with and without dilutions of SW872-conditioned media was assessed using CyQuant assay as described in Materials and Methods. Results are expressed as mean ± SE from 3 separate experiments. *Significantly different from the control ($P < .05$). OD indicates optical density; HuLM cells, human uterine leiomyoma cells; SE, standard error.

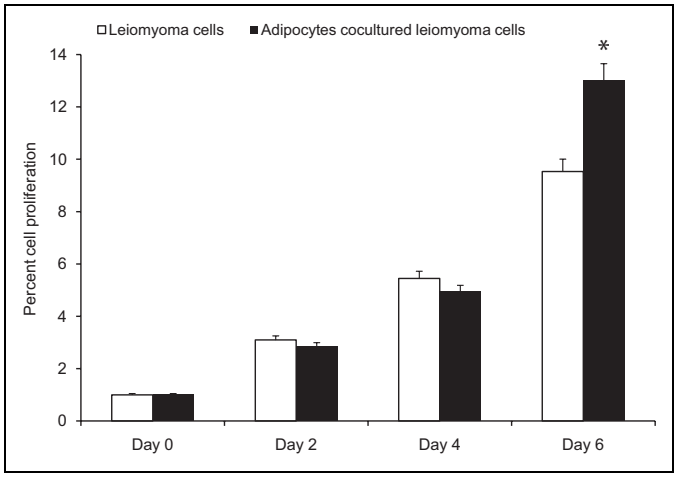

Figure 2. HuLM cell proliferation with and without SW872 coculture (mean ± SE; n = 3). HuLM cells were cocultured with SW872 cells for days 2, 4, and 6. Comparison in cell proliferation is made with HuLM cells grown without SW872 coculture. Proliferation in HuLM cells was measured using CyQuant assay. Results are expressed as mean ± SE from 3 separate experiments. *Significantly different from the control ($P < .006$). HuLM cells indicate human uterine leiomyoma cells; SE, standard error.

The control group consisted of HuLM cells without coculture of SW872 cells. The treatment group consisted of HuLM cells and SW872 cocultured cells using the transwell system till day 6. As shown in Figure 2, a progressive increase in the number of HuLM cells was observed with time in adipocytes-cocultured cells compared to the control. SW872 adipocytes-cocultured leiomyoma cells increased by about 13% ± 0.05% on day 6 compared to control cells ($P < .006$).

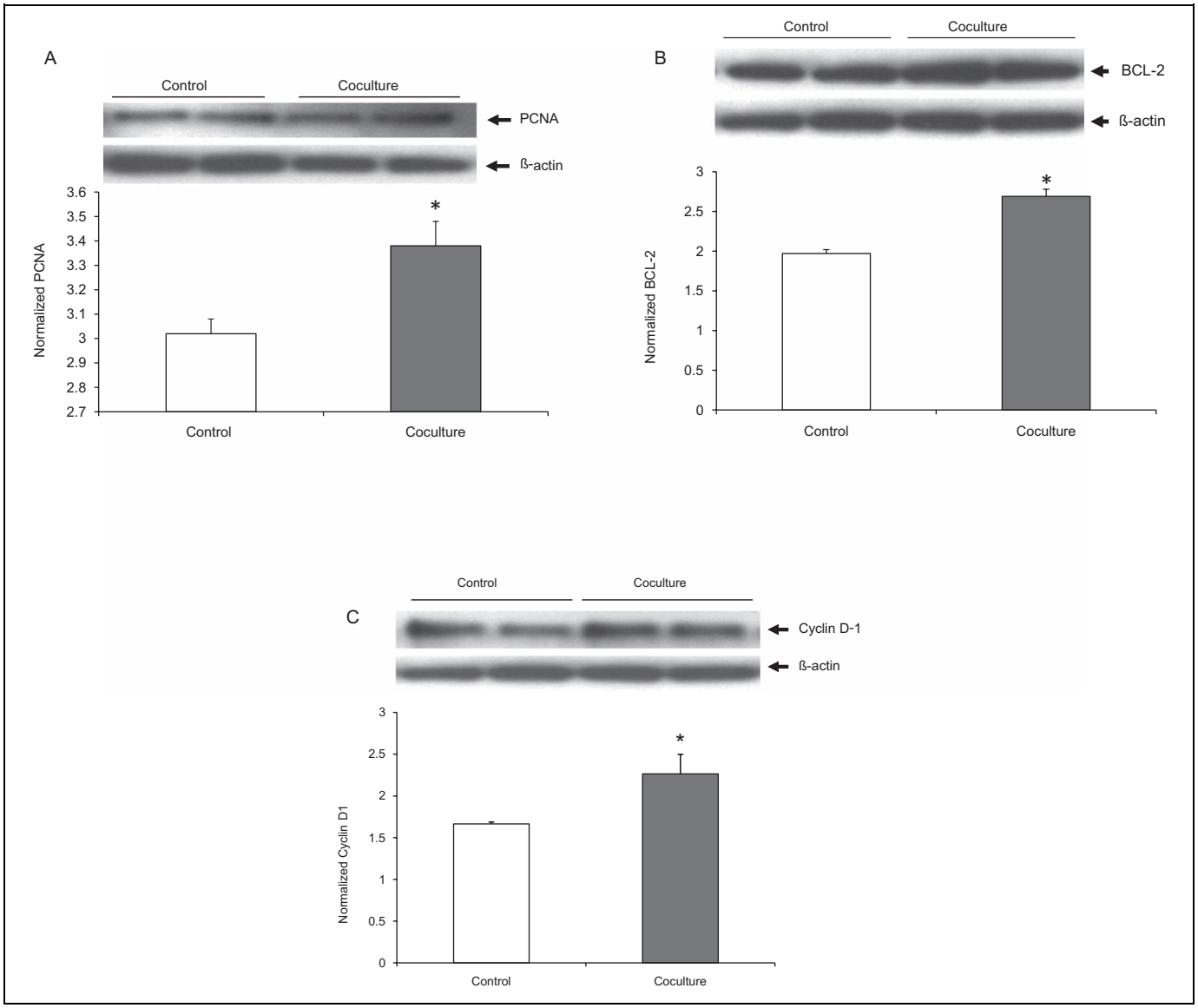

Figure 3. Western blot analysis of PCNA, BCL-2, and cyclin D1 in HuLM cells with and without coculture. HuLM and SW872 cells were cocultured for 6 days. Lysates prepared from control and cocultured cells were analyzed by Western blotting with (A) anti-PCNA, (B) anti-BCL-2, and (C) anti-cyclin D1 antibodies. The intensity of each protein signal was quantified and normalized with the corresponding β-actin. Results shown represent 3 separate experiments with comparable results. *$P < .05$ compared with control. HuLM cells indicate human uterine leiomyoma cells; PCNA, proliferating cell nuclear antigen; BCL-2, B-cell lymphoma 2.

Adipocyte-Coculture Modulates Expression of Protein Markers in Human Leiomyoma Cells

Western blot assay was performed to detect changes in the expression of various protein markers: cell proliferation (PCNA), antiapoptosis (BCL-2), and cell cycle division (cyclin D1). Proliferating cell nuclear antigen expression in HuLM cells cocultured with SW872 cells increased significantly, by approximately 12.0% ($P < .03$; Figure 3A). Expression of antiapoptotic protein marker BCL-2 showed a marked 36% increase ($P < .0001$; Figure 3B). Expression of cyclin D1, a marker for the conversion of cells from S to G phase, was significantly increased by 36% ($P < .002$; Figure 3C). The induction of cell proliferation and antiapoptosis genes indicates a positive influence of the SW872 cells on the HuLM cells.

Adipocytes-Derived Effects on Human Leiomyoma Cells Are Mediated Via TNF-α

We next wanted to characterize the nature of the humoral factors that possibly mediate these marked effects of SW872 cells on HuLM cells. Numerous adipokines have been described in the literature,[10,11,28,29] of which TNF-α is perhaps the most important and widely studied.[10,11,30] Thus, we evaluated the role of TNF-α in mediating the proliferatory effects of

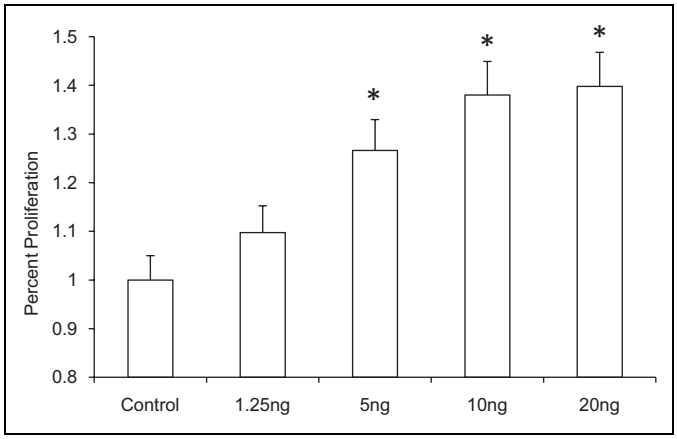

Figure 4. Effect of different concentrations of TNF-α on HuLM proliferation. Cell proliferation in HuLM treated with different concentrations of TNF-α was assessed using CyQuant assay as described in Materials and Methods. Results are expressed as mean ± SE from 3 separate experiments. *Significantly different from the control ($P < .05$). TNF-α indicates tumor necrosis factor α; HuLM cells, human uterine leiomyoma cells; SE, standard error.

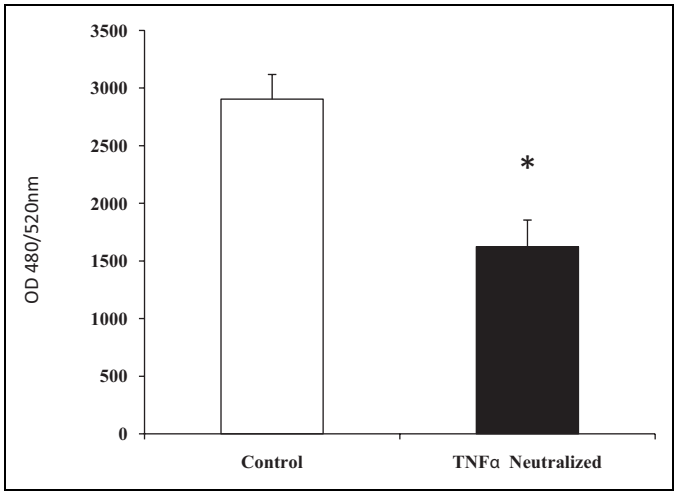

Figure 5. Effects of anti-TNF-α-neutralizing antibodies on HuLM cell proliferation. Conditioned media collected from SW872 cells grown to more than 80% confluence was centrifuged, filtered, and diluted to 1%. This conditioned media was treated with 1 ng/mL of anti-TNF-α antibody for an hour at 37°C. The neutralized conditioned media was then added to HuLM cells and cell proliferation measured using CyQuant assay. Results shown represent 3 separate experiments with comparable results, *$P < .05$ (mean ± SE; n = 3). OD indicates optical density; TNF-α, tumor necrosis factor α; HuLM cells, human uterine leiomyoma cells; SE, standard error.

SW872 adipocytes on HuLM cells. Human uterine leiomyoma cells were treated with different concentrations of TNF-α (1.25, 2.5, 5, and 10 ng) for 72 hours and cell proliferation was determined using CyQuant assay (Figure 4). We observed that all concentrations higher than 2.5 ng showed a statistically significant increase in cell proliferation in comparison to control ($P < .05$). This suggested that TNF-α induces cell proliferation, and this growth is dependent on the concentration of TNF-α. Elimination of TNF-α in the SW872-conditioned media using anti-TNF-α-neutralizing antibody essentially reversed most of the proliferatory effects in the SW872 cocultured leiomyoma cells. Cell proliferation was attenuated by 35% in the antibody-neutralized cocultured HuLM cells ($P < .05$; Figure 5), while PCNA and cyclin D1 expression did not show significant differences between the control and cocultured human leiomyoma cells with TNF-α-neutralizing antibodies. B-cell lymphoma 2 showed a 25% reduced expression in cocultured, antibody-neutralized group compared to the control group ($P < .018$; Figure 6).

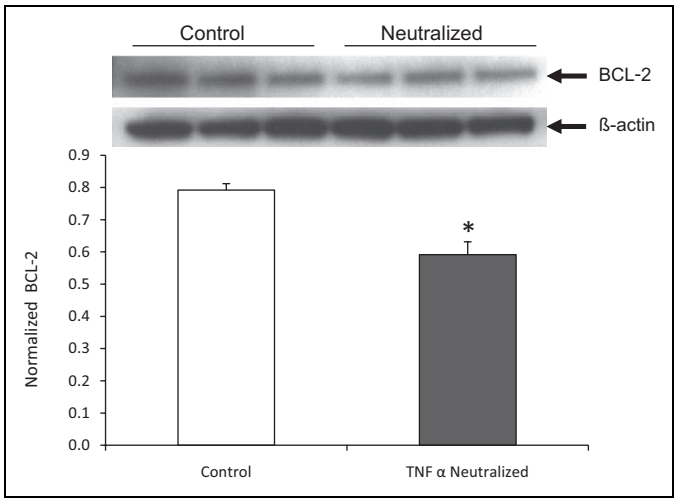

Figure 6. Effects of anti-TNF-α-neutralized conditioned media on the expression of anti-apoptotic BCL-2 in HuLM cells. Conditioned media collected from SW872 cells grown to more than 80% confluence was centrifuged, filtered, diluted to 1%, and neutralized for an hour with 1 ng/mL of anti-TNF-α antibody at 37°C. Lysates prepared from control and treated HuLM cells were analyzed by Western blotting with anti-BCL-2 antibody. The intensity of each protein signal was quantified and normalized with corresponding β-actin. *$P < .05$ compared with control (mean ± SE; n = 3). TNF-α indicates tumor necrosis factor α; HuLM cells, human uterine leiomyoma cells; BCL-2, B-cell lymphoma 2; SE, standard error.

Discussion

Obesity, a major risk factor for uterine fibroids, is defined as a state of low-grade chronic inflammation with increased levels of inflammatory markers.[9,7,31] Higher levels of TNF-α, a prominent inflammatory cytokine, is found in obese women.[18] Obesity-related chronic inflammation has been linked to initiation and progression of several cancers.[8,32,33] The risk of fibroid formation increases by 21% with every 10 kg increase in body weight.[34] The mechanism of this association is unclear and a biological interaction between adipose cells and leiomyoma cells has not yet been demonstrated. In this study, we evaluated the association using a coculture model with representative cell lines of adipocytes and human uterine leiomyoma cells. The results indicate that our coculture system provides a suitable in vitro experimental model to study this interaction.

We demonstrated that coculture of adipocytes and uterine leiomyoma cells results in an increased proliferation of leiomyoma cells. We also demonstrated that TNF-α treatment increases HuLM cell proliferation in a concentration-dependent manner.

Various growth factors and hormones have been reported to increase uterine leiomyoma cell proliferation.[1,36] In this study, increased proliferation of uterine leiomyoma cells in SW872-conditioned media as well as coculture suggests a propagative influence of SW872 cells. Our invitro coculture model demonstrates that specific biologically active growth factors secreted by SW872 cells into the media have a proliferative effect on the uterine leiomyoma cells. Cocultured leiomyoma cells showed an upregulation of PCNA, cyclin D1, and BCL-2 protein, which further confirms the secretion and role of the soluble factors released from SW872 cells in mediating cell proliferation in leiomyoma cells.

Tumor necrosis factor α (TNF-α) is a multifunctional cytokine eliciting responses of both apoptosis and proliferation in different cell types.[37,38] We found that neutralization of SW872 cells–conditioned media with anti-TNF-α antibody reduced proliferation in HuLM cells. This indicates that TNF-α secreted by the SW872 cells increase leiomyoma cell proliferation and therefore TNF-α secreted from adipose tissue may play an active role in uterine leiomyoma tumorigenesis in vivo. Adipose tissue is reported to secrete TNF-α both locally and into the circulation,[39–41] while other studies report that TNF-α downregulates apoptosis in activated hepatic stellate cells.[42] In our studies, HuLM cells cultured in TNF-α-neutralized SW872-conditioned medium showed a reduced expression of antiapoptotic gene BCL-2. This confirmed the role of TNF-α in the reduction of HuLM apoptosis. Similar reduction of apoptosis by TNF-α via upregulation of NFkB and BCLxl has been reported in hepatic stellate cells.[42] Our findings indicate a major role of TNF-α in HuLM proliferation probably mediated by regulation of downstream proteins involved in apoptosis. Further studies are needed to identify and characterize adipokines with similar effects on uterine leiomyoma cells and investigate their mechanism of action.

An in vitro coculturing system is a useful model to gain a better understanding of cell–cell interactions that may occur in vivo.[43] The study of molecular mechanisms of cell interaction is highly complicated in vivo due to many variables. Overall observations noted in the cell proliferation and gene expression in this study may not be dramatic. The cause for this may be attributed to many reasons including innate cell characteristics, low cell number due to restricted space in the coculture system, amount of adipokine secreted, and absence or presence of other cofactors/inhibitors which may have a detrimental effect on the coculture model used here. Furthermore, the specialized culture medium required by leiomyoma cells and the nature of SW872 cells being a liposarcoma cell line may also affect the magnitude of results observed in this study. Use of coculture has many limitations as well as advantages; this study has indicated that adipocyte cells have a stimulatory effect on leiomyoma cells. In conclusion, our study demonstrates that human SW872 cells stimulate proliferation of human leiomyoma cells which is modulated by proinflammatory cytokine TNF-α, secreted by the SW872 cells. Further studies are warranted to elucidate the potential implication of the findings presented here, which suggest the role of obesity-induced inflammation as a risk of uterine leiomyoma.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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